ORGANOSULFUR GARLIC-DERIVED COMPOUNDS INDUCE ABCA1 GENE EXPRESSION IN RAW 264.7 CELLS

By

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EXPRESSION IN RAW 264.7

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Abstract: Cardiovascular disease (CVD) is currently the leading cause of death in the United States [1]. One of the key factors in CVD development is cholesterol levels; accumulation of cholesterol in the macrophage has been linked to plaque formation and subsequent heart attack or stroke [3]. ATP-binding cassette transporter A1 (ABCA1) is the protein that facilitates cholesterol efflux out of the macrophage, and increasing this efflux can prevent the macrophage from being a pro-atherogenic foam cell. In addition, garlic-derived organosulfur compounds have been suggested to have cholesterol-lowering effects [35]. The aim of this study was to investigate the effects in mouse macrophages of garlic compounds diallyl disulfide (DADS), allyl mercaptan (AM), and S-allyl-L-cysteine (SAC) on ABCA1 expression compared with trichostatin A (TSA), a drug known to induce ABCA1 via histone modification. RAW 264.7 cells were treated with these compounds for six hours, and RNA was isolated, reverse transcribed, and analyzed for changes in gene expression. ABCA1 expression was induced with DADS, AM, and SAC, but no change was seen in expression of ATP-binding cassette transporter G1 (ABCG1) or HMG Co-A Reductase (HMGCR). Chromatin immunoprecipitation assays showed no significant differences in acetylated histone H3 or H4 in the promoter of ABCA1 due to treatment with DADS. Luciferase reporter assays revealed garlic-induced ABCA1 promoter activation with fragments as short as 75 bp upstream of the transcriptional start site and up to 2500 bp, suggesting the transcriptional upregulation may not be due to histone modification in the promoter. These results show that ABCA1 mRNA is significantly increased by treatment with DADS, SAC, and AM, and this effect may or may not be mediated by histone modification. Additional studies are required to understand the mechanisms underlying this effect.

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CHAPTER I

INTRODUCTION

Over the last several decades, America has seen a significant shift in patterns of diet and physical activity. Changes such as increasing consumption of a high-fat, high-sugar diet and decreasing levels of physical activity have driven an increase in prevalence of metabolic disorders such as cardiovascular disease, obesity, and diabetes. Cardiovascular disease (CVD) is currently the leading cause of death in the United States [1]. Oklahoma is no exception to this trend; 10,000 Oklahomans died in 2014 due to CVD alone [2]. Due to these trends, prevention and treatment of CVD have become a priority in national health. In the interest of using lifestyle approaches to treat CVD, functional properties of foods such as fruits and vegetables are being explored in hopes of finding new strategies to reduce the impact of this disease.

Serum cholesterol levels are a main target of CVD treatment due to the potential of this cholesterol to be transported into the macrophage and accumulate. This buildup can eventually lead to plaque formation and subsequent heart attack or stroke. High density lipoprotein (HDL), known as the "good cholesterol," is involved in the reverse export of cholesterol from macrophages back to the liver, potentially reversing the buildup of cholesterol and slowing CVD development. ATP-binding cassette transporter A1 (ABCA1), a membrane-bound protein in macrophages, facilitates this process, along

with its partner ABCG1. Increasing ABCA1 and ABCG1 abundance is a potential strategy for lowering cholesterol levels as a complement to current cholesterol-lowering medications [14], which target the cholesterol synthesis pathway but may also have undesirable side effects such as increasing risk of developing type 2 diabetes [22].

Garlic, or *Allium sativum*, has long been claimed to possess extensive health benefits including immune boosting, antioxidant, antidiabetic, and hepatoprotective [26]. Consumption of a garlic supplement in various forms for at least two months in individuals with elevated total cholesterol >200 mg/dL was associated with an 8% reduction in total cholesterol with only a slight improvement in serum HDL and no effect on triglyceride levels [35]. This study demonstrates the potential of garlic to be part of the therapeutic strategy for treating high cholesterol. More specifically, the organosulfur compounds that make up 2.3% of raw garlic are potentially bioactive. Compounds of interest include diallyl disulfide (DADS), allyl mercaptan (AM), and S-allyl-L-cysteine (SAC).

Research in the cancer field has suggested that these compounds may work by inhibition of histone deacetylases (HDAC) [46-48]. HDACs work to deacetylate histone proteins in the scaffolding of nuclear DNA, typically leading to a decrease in transcription due to a tighter chromatin structure. Thus, an HDAC inhibitor (HDACi) can lead to an increase in transcription by allowing the chromatin to remain open. Garlic-derived compounds have been shown to have HDACi function in affecting genes related to cancer.

Objective: The present study was undertaken to investigate the effects of garlic-derived compounds on CVD-related genes and possible mechanisms for these effects.

Hypothesis: Garlic-derived compounds DADS, AM, and SAC increase mRNA expression of ABCA1 in macrophages, and may be exerting this effect by working as HDACi that modify histones in the promoter region of ABCA1.

Aim 1: To distinguish the effects of garlic-derived compounds on transcription regulation of genes ABCA1 and ABCG1.

Aim 2: To explore the role of garlic-derived compounds in chromatin remodeling in the promoter region of ABCA1 via HDAC inhibition.

Aim 3: To characterize the specific region of the ABCA1 promoter that is activated in response to garlic-derived compound treatment.

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CHAPTER II

REVIEW OF LITERATURE

CVD pathophysiology

CVD can occur as a result of atherosclerosis, a condition in which plaque formation in blood vessel walls leads to blockages and subsequent strokes and/or heart attacks. Atherosclerosis is defined broadly as a "response to injury" [3]. This injury occurs in a blood vessel wall in response to a variety of factors, including high blood pressure, smoking, diabetes, and elevated blood lipid levels. These factors can cause arterial endothelial cells to express adhesion molecules that then retain monocytes streaming through the blood. Once inside the artery wall, the monocytes differentiate into macrophages. As the endothelium becomes increasingly permeable, cholesterol-rich low density lipoprotein (LDL) particles can also enter and be phagocytosed by macrophages. Cholesterol-rich macrophages are termed "foam cells." Other changes in the arterial wall include the uptake of smooth muscle cells (SMCs), increased synthesis of proteins such as collagen, and cytokine secretion [4]. Foam cells and SMCs can die, causing a buildup in a central lipid core, or plaque region. Eventually this plaque can rupture, leading to the attraction of platelets and other coagulation factors, and blockage of blood flow can cause a heart attack or stroke.

Lipids and cholesterol play a significant role in disease development, and are transported through the blood bound to lipoproteins. Low density lipoprotein (LDL) transports lipids and cholesterol from the liver to the rest of the body and can be found circulating in the blood at varying levels. CVD has been associated with elevated levels of circulating cholesterol and LDL [5], and one cell type affected by these high levels is the macrophage. While the macrophage is normally thought of in terms of immunity due to its role in phagocytosing foreign invaders and protecting the body, it also has the ability to take in large amounts of cholesterol bound to LDL. When a macrophage takes up more cholesterol than it can efflux to HDL, cholesterol is stored in the cytoplasm in the form of cholesteryl ester. Accumulating levels of cytoplasmic cholesterol turn macrophages into foam cells, which then and contribute to the buildup of plaque in atherosclerotic lesion. Uptake of oxidized LDL causes macrophages to produce pro-inflammatory signaling molecules, or cytokines, which speed the development of atherosclerosis [6]. Thus, controlling blood lipid and cholesterol levels through dietary and other therapeutic interventions is a significant regulatory action in preventing the development of CVD. Indeed, a meta-analysis of 38 relevant studies showed that a 10% reduction in serum cholesterol can reduce CVD risk by as much as 54% [7].

Another lipoprotein, high density lipoprotein (HDL), performs an action known as reverse cholesterol transport (RCT), in which cholesterol is transported from peripheral cells back to the liver [8]. Here, it contributes to a pool of cholesterol that can be used to synthesize new bile acids. Through the efflux process, cholesterol levels in peripheral cells, specifically macrophages, can be decreased. Dysfunction of this process can contribute to atherosclerotic foam cell development. Low serum HDL has been

implicated in CVD development [9], although it remains to be determined whether this is a correlation or causality. In addition to testing serum HDL, recent studies have also begun to explore the cholesterol efflux capacity of HDL [6]. A longitudinal study following a large group cohort of initially healthy adults for nine years found that lowered cholesterol efflux capacity, as evidenced by plasma uptake of fluorescence-labeled cholesterol, was positively associated with incidence of cardiovascular disease [10]. These results suggest that increasing the capacity of the macrophage to efflux cholesterol to HDL may reduce CVD risk.

ABCA1 in reverse cholesterol transport

ATP-binding cassette transporter A1 (ABCA1) is the membrane-bound protein that facilitates reverse cholesterol efflux in peripheral cells, specifically macrophages [11]. ABCA1 binds to the circulating lipoprotein apoA-1 (also known as pre-β-HDL) and facilitates transport of unesterified cholesterol from the macrophage to apoA-1. After receiving this cholesterol, apoA-1 becomes an HDL particle. Alternate efflux pathways involve ATP-binding cassette transporter G1 (ABCG1) and scavenger receptor B type I (SR-BI), which both efflux cholesterol to mature HDL particles. HDL carries this cholesterol to the liver, where a hepatic form of SR-BI takes up the cholesterol. In the liver, the cholesterol can be incorporated into bile acids [12].

Mutations in ABCA1 are known to cause Tangier disease, where a virtual absence of HDL leads to an accumulation of cholesterol and subsequent hepatosplenomegaly, peripheral neuropathy, and increased CVD risk [13]. Increased ABCA1 expression can lead to increased RCT, a process that can prevent macrophages from becoming pro-

atherogenic foam cells. Indeed, overexpression of ABCA1 in LDL-receptor knockout mice was shown to inhibit atherosclerotic lesion progression [14]. The half-life of ABCA1 mRNA and protein is relatively short (no longer than four hours), signifying the efflux process is quickly responsive to changes in signaling [15]. Thus, ABCA1 is a significant control point in overall cholesterol metabolism. Importantly, portions of the ABCA1 promoter region are conserved between humans and mice (**Figure 1**).

The most well-characterized form of ABCA1 regulation is the LXR pathway. LXR, or liver X receptor, is a nuclear nutrient sensor present throughout the body in two isoforms: LXRα and LXRβ. LXRα is expressed in the liver, kidney, intestine, and macrophage; LXRβ is ubiquitously expressed [16]. Oxysterol, an oxidized derivative of cholesterol, is ligand that activates LXRs. When cellular cholesterol levels are high, oxysterol binds to LXR, which then binds to its heterodimer, retinoid X receptor (RXR). This complex binds to the ABCA1 promoter to increase transcription and promote cholesterol efflux. Through this signaling system, LXR acts to counteract higher cell cholesterol levels. Thus, LXR agonists have been a suggested therapeutic drug for increasing RCT and treating CVD. However, the LXR signaling pathway also triggers an increase in lipid synthesis and higher plasma triglycerides [17], which would be counterproductive for patients with pre-existing hyperlipidemia.

The LXR binding region is located 50 bp upstream from the ABCA1 transcriptional start site (**Figure 1**). Other important binding factors in the ABCA1 promoter include specificity protein 1 (Sp1, -100 and -166 bp), and activator protein 1 (AP-1, -131 bp). Sp1 is a ubiquitously expressed transcription factor known to affect cell cycle regulation genes, housekeeping genes, and tissue-specific genes. A variety of

Figure 1.

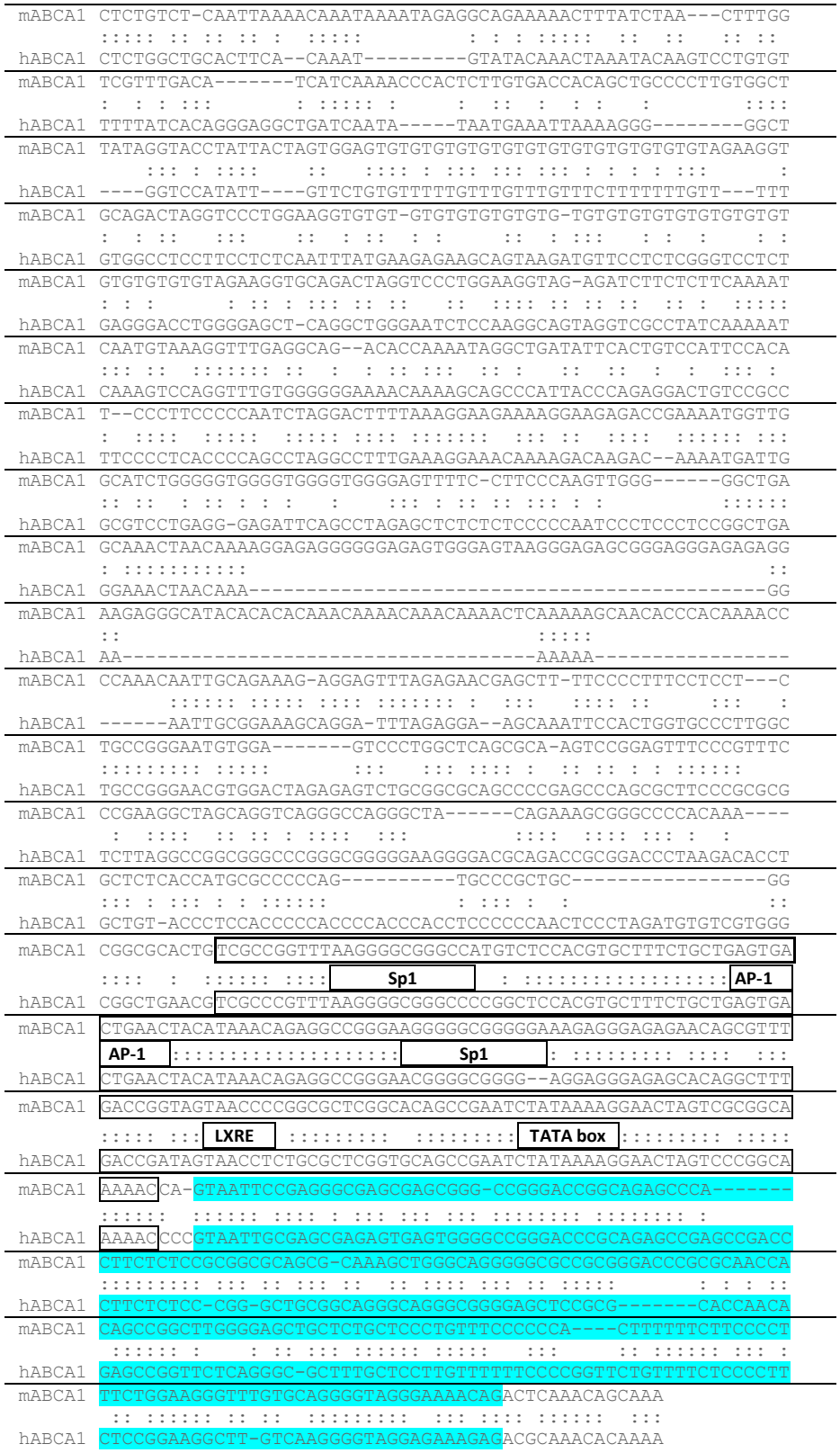


Figure 1: Comparison of the mouse and human ABCA1 promoter sequences. BLAST and LAlign software were used to align homologous sequences (connected by a colon) in the 5' flanking region of the ABCA1 gene, from -2500 bp to +300 bp. Analysis revealed a 189 bp sequence (boxed) with 90% similarity in the region immediately preceding the conserved transcriptional start site of ABCA1, which suggests a conserved promoter for ABCA1 exists in this region [18]. Other conserved regions include the TATA box (-35 bp), LXRE (-53 bp), Sp1 (-100 and -166 bp), and AP-1 (-131 bp). The transcriptional start site and first exon of ABCA1 are highlighted.

proteins interact with Sp1 to either increase or decrease transcription, including estrogen receptor proteins (increase) and p53 (decrease) [19], and aberrant Sp1 function has been implicated in cancers and neurodegenerative diseases. Sp1 binding to the ABCA1 promoter was found to be essential for LDL-induced upregulation of ABCA1 expression [16]. Similarly, AP-1 is a transcription factor linked to cellular proliferation and apoptosis, and its control of expression of cytokines such as tumor necrosis factor alpha and interleukin 1 implicate it in progression of immune disorders and cancers [20]. No studies to date have documented a role of AP-1 in ABCA1 regulation.

Prevention of CVD

Due to the importance of blood lipid levels in CVD, many drug therapies for CVD involve lipid-lowering medications. The main class of these drugs is termed "statins," which act by inhibiting key enzymes in the cholesterol biosynthesis pathway (namely 3-hydroxy-3-methylglutaryl-CoA reductase, or HMGCR). However, use of statins has been linked to complications such as muscle weakness and damage, peripheral neuropathy, and an elevated risk of developing type 2 diabetes [21, 22]. Due to these risks, alternative approaches to treating and preventing CVD must be explored.

Research has linked the prevention of CVD to several key lifestyle factors, including avoidance of smoking, regular physical activity, normal body mass index, and eating a balanced diet [23]. Dietary recommendations include limiting intake of trans fats and saturated fats, sugar-sweetened beverages, and refined carbohydrates, and increasing consumption of fruits, vegetables, whole grains, and unsaturated fats. The popular Dietary Approaches to Stop Hypertension (DASH) Diet advocates these

recommendations along with limiting sodium intake [24], and adherence to this diet has been shown to significantly reduce CVD risk [25].

Increasing consumption of "whole foods" (fruits, vegetables, whole grains, nuts, and legumes) as an approach to reduce risk of CVD is an attractive alternative to drug therapies due to its accessibility to the general public and lack of dangerous side effects. One such food, garlic, has received recent attention for its role in reducing CVD risk.

Garlic

Garlic (*Allium sativum*) is a food belonging to the onion genus and has long been touted for its potential to prevent disease. As long as 3,500 years ago, an Egyptian medicinal guide advocated consumption of garlic to treat heart disorders, tumors, and worms [26]. Anecdotal evidence promoting garlic's health benefits has accumulated over the years, and through scientific research it has become clear that the bioactive sulfur-containing compounds in garlic drive these benefits.

Garlic naturally contains 2.3% organosulfur compounds, which create its distinctive odor and taste; however, these compounds are volatile and can change forms (**Figure 2**). In an intact garlic bulb, the major sulfur-containing compounds are γ-glutamyl-S-allyl-L-cysteine and S-allyl-L-cysteine sulfoxides, or alliin [27]. Alliin is converted into thiosulfinates such as allicin through enzymatic action when the garlic bulb is crushed or cut (**Figure 2**). γ-Glutamyl-S-allyl-L-cysteine, on the other hand, is converted into S-allyl-L-cysteine (SAC) when an aqueous solution is used to extract garlic [27].

The compound allicin is not absorbed by the human body [28] and has been found to be unstable: after 20 hours at 20°C, it completely degraded into diallyl disulfide (DADS) (66%), diallyl sulfide (DAS) (14%), and diallyl trisulfide (DATS) (9%) [29]. It is believed that stomach acid degrades allicin to these compounds that may then be absorbed by humans and animals. Once absorbed, DADS circulates in the body along with its major metabolite, allyl mercaptan (AM); in rat liver cells DADS was converted to AM within 30 minutes after ingestion [30]. Indeed, after ingestion of grated garlic, a breath test administered to human subjects showed two major peaks identified as AM and DADS [31].

Preparation of garlic can greatly affect the composition of bioactive components, making it difficult to elucidate the effects of single compounds in studies involving animal or human dietary interventions. Garlic powder, garlic oil, and aged garlic extract (AGE) are commonly used preparations. Garlic powder and garlic oil contain oil-soluble sulfur compounds such as DADS and DATS [27]. Aged garlic extract (AGE) refers to sliced raw garlic stored in alcohol for 15-20 months, which creates an overall loss of allicin and high levels of SAC [32], which can be absorbed by the human body [33].

The roles of these bioactive components in health are being actively explored in both humans and animals. Suggested physiological roles include antioxidant, immune boosting, antimicrobial, antidiabetic, hepatoprotective, and anti-platelet aggregatory [25], but arguably the most interest has been in the realms of cancer and CVD.

Figure 2.

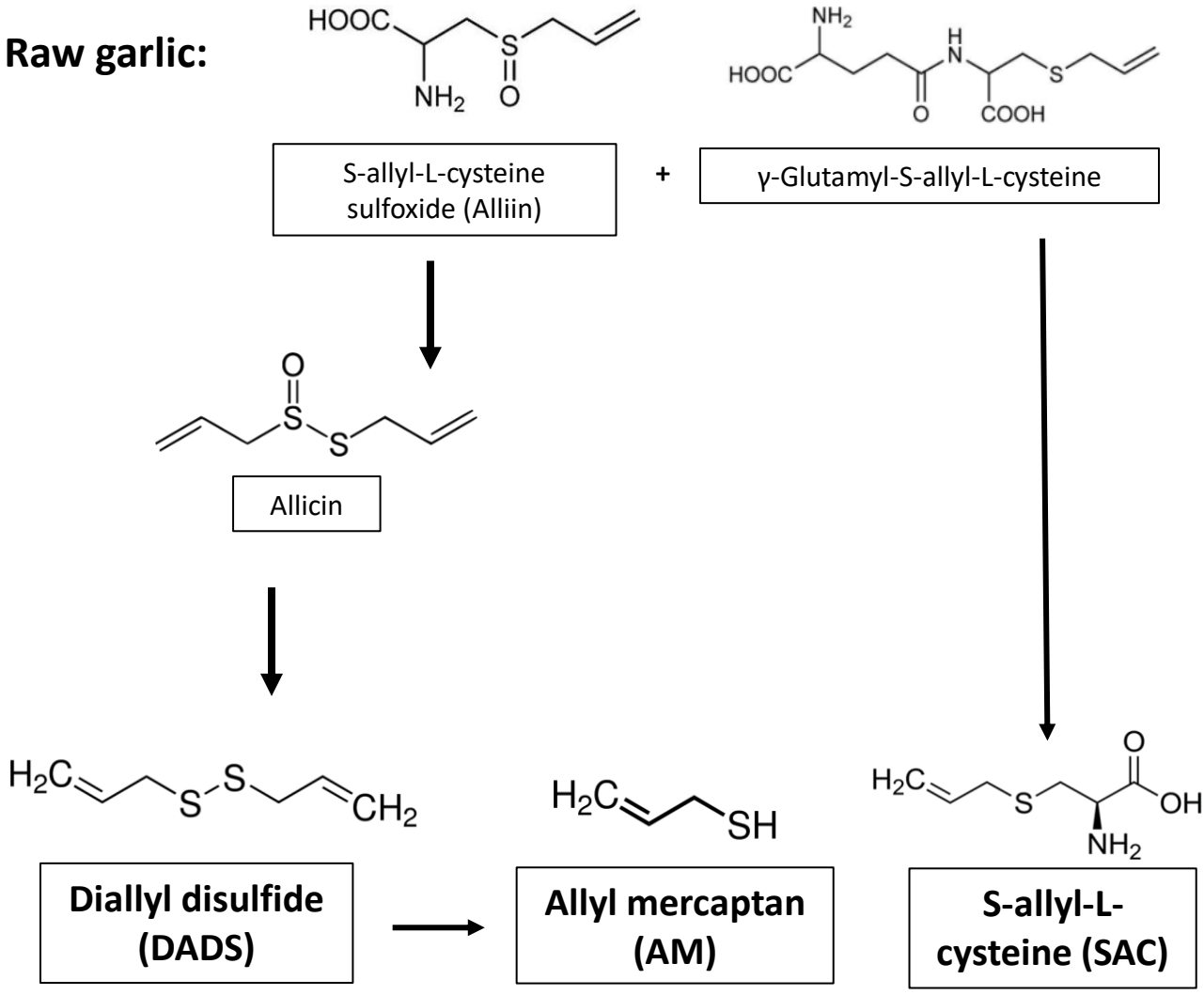


Figure 2: Garlic compound structures and decomposition pathways. γ-glutamyl-S-allyl-L-cysteine and S-allyl-L-cysteine sulfoxides (alliin) are present in raw, intact garlic [34]. Alliin is converted to allicin when the bulb is crushed or cut, but quickly decomposes to DADS. In the body, DADS can be metabolized to AM. γ-Glutamyl-S-allyl-L-cysteine is converted into S-allyl-L-cysteine (SAC) in aqueous garlic extracts.

Garlic in promoting RCT/decreasing cholesterol synthesis

Much research has been done in regards to garlic's effect on CVD related to serum lipids. A recent meta-analysis of 39 trials testing the effect of garlic on total cholesterol, LDL, HDL, and triglycerides [35] found an overall 8% reduction in total cholesterol related to garlic treatment, which is associated with a 38% decrease in risk of a coronary event [7]. Garlic was also found to moderately lower LDL and raise HDL, and Reid suggests that although the cholesterol-lowering effects of garlic may be modest, the absence of serious side effects related to this treatment makes garlic a viable alternative to traditional cholesterol-lowering medications [35].

Although a sizeable amount of research has been done on the correlation between garlic treatment and cardiovascular outcomes including serum lipoprotein levels [33-36], research is less abundant in regards to the mechanism of action. Cho et al. [36] found that allyl mercaptan (AM) decreased cholesterol synthesis and secretion in human Hep-G2 cells, as evidenced by levels of radiolabeled [^{3}H] acetate incorporation into cholesterol and subsequent secretion into media. This suggests garlic compounds may help by reducing overall cholesterol synthesis. Lee et al. [37] found that rats supplemented with a garlic extract on a high-fat diet had higher serum HDL cholesterol related to increased gene expression of apolipoprotein A-I. While both are viable pathways, limited research suggests that garlic might also work to improve cardiovascular outcomes by increasing reverse cholesterol transport through the ABCA1 transporter. Malekpour et al. [38] found that human THP-1 macrophages treated with SAC had increased mRNA expression of ABCA1.

Epigenetic modification & histone remodeling

While one line of our research involves looking at changes in gene expression due to garlic compound treatment, a deeper question involves the molecular mechanism by which these changes occur. A feasible explanation is that garlic works through epigenetic modification. "Epigenetics" refers to alterations of gene expression that leave the actual DNA sequence unchanged. Excitement related to the field of epigenetics has risen as research unfolds because epigenetic abnormalities are much more modifiable than genetic abnormalities. Treatments for chronic diseases that target gene expression via epigenetic mechanisms are more attractive than altering the actual DNA sequence; the same effect can be accomplished by "silencing" a gene rather than permanently modifying it.

One pathway to epigenetic modification involves a class of regulatory proteins known as histones. Histone proteins act as a scaffolding around which double-stranded DNA is wrapped within the chromosome structure. In the past it was thought that histones played only a structural role, but more recent research shows that modification to histones can have a dramatic effect on DNA transcription [39]. These modifications are not permanent, so genes can be alternatively activated or repressed depending on the system doing the modifications [46]. The most significant way histones can be modified is acetylation/deacetylation, which involves the "relaxing" of the DNA wound around the histone. This in turn can cause increased transcription. When the acetyl group is removed, the DNA is wound more tightly and is less accessible to transcription enzymes.

The modifications to histones are temporary, and enzymes are required to maintain them. Histone acetyltransferases (HATs) transfer acetyl groups to the histones;

histone deacetylases (HDACs) remove these groups. The action of HDACs is correlated with a transcriptional repression due to the tighter chromatin structure. Eighteen known HDACs exist in mammals and are divided into four classes. Class I and II (HDACs 1-10) are associated with adaptive immunity, and functions include growth and development, immune/inflammatory response, and endothelial integrity [46]. Class III (HDACs 12-18) are known as SIRTs and act in the mitochondria via NAD^+-dependent deacetylation; functions include cellular aging, transcription, DNA repair, and apoptosis [46]. Class IV includes only HDAC 11, and dysregulation is associated with gene variants (rather than overexpression) in multiple sclerosis [40].

HDACs naturally function to regulate many cellular processes, including adipogenesis, lipid metabolism, myogenesis, and gluconeogenesis. In a healthy individual, HDACs inactivate genes at appropriate times. For example, HDAC3 works to repress lipid oxidation during the fed state [41]. However, HDAC dysfunction has been implicated in the pathogenesis of various diseases including cancer [46]. Overexpression of HDAC may lead to lower transcription of tumor suppressor genes and contribute to the advancement of the disease. HDAC activity has been linked to progression of cancers such as prostate, gastric, and colorectal [42, 43, 34]. HDAC3 and 7 are associated with inflammatory gene expression and pro-inflammatory activation of macrophages [44, 45], and HDAC9 deletion in $LDLr^{-/-}$ mice led to a decrease in atherosclerotic lesion size, enhanced macrophage cholesterol efflux, and a reduction in total and LDL cholesterol [66].

Due to the potentially harmful effects of some HDACs, compounds that have an inhibitory effect on HDACs (called HDAC inhibitors, or HDACi) are of interest. Both

synthetic and naturally occurring HDACi exist. A commonly used HDACi is trichostatin A (TSA), which is a naturally occurring microbial metabolite of *Streptomyces hygroscopicus* [46]. TSA is a pan-HDAC inhibitor and is extremely potent. The main effect of treatment with HDACi is a de-repression of transcription, leading to increased gene expression. HDACi have been suggested to reactivate genes normally silenced in cancer cells such as those involved in normal cell death; indeed, HDACi were shown to induce apoptosis in a range of cancer cells [46].

The field of cancer research offers valuable insight into the possible mechanism behind the effects of the bioactive components of garlic. Nian et al. [47] showed that treatment of human HT-29 colon adenocarcinoma cells with garlic compound allyl mercaptan (AM) inhibits activity of HDAC and increases histone acetylation, specifically in the promoter region of P21WAF1, a gene related to cell cycle control. Levels of Sp3, a transcription factor for P21WAF1, are higher in AM-treated cells. This study suggests that AM works to increase expression of tumor suppressor genes via HDACi action. Druesne et al. [48] demonstrated that treatment with diallyl disulfide (DADS) has a similar effect in these cells; specifically, it induces histone hyperacetylation at histone H3, lysine residue 14.

Extensive research has been conducted on bioactive compounds of garlic in genes related to cancer, but fewer investigations have been done regarding CVD. Thus, we proposed to explore the possible roles of garlic compounds in increasing expression of cholesterol efflux transporter ABCA1, as well as secondary efflux transporter ATP-binding cassette transporter G1 (ABCG1) and HMG Co-A Reductase (HMGCR). A chromatin immunoprecipitation assay was also used to explore possible histone

modification in the ABCA1 promoter due to garlic compound treatment. Finally, we developed a gene construct system using a luciferase reporter assay to investigate what specific part of the ABCA1 promoter might be modified by garlic compound treatment, and whether this treatment could still modify transiently transfected plasmids not associated with chromatin structure.

CHAPTER III

METHODOLOGY

Maintenance of cells

Murine macrophage RAW 264.7 cell line was obtained from a subclone of Abelson murine leukemia virus-induced tumor (ATCC; #TIB-71, Manassas, VA). RAW 264.7 cells were maintained in complete media containing Dulbecco's Modified Eagle's Medium (DMEM) with 4.5g/L glucose, without L-glutamine, and with sodium pyruvate (VWR; Radnor, PA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals; Flowery Branch, GA), 1% penicillin-streptomycin/L-glutamine (PS/L-glut) (Corning; Corning, NY). The cells were grown to 80% confluence in a 100-mm plate in 10 mL of complete media and maintained at 37°C in a humidified incubator. Cells were passaged up to 20 times at a frequency of 48 hours at a 1:20 dilution. To determine the concentration of viable cells, cells were stained with 0.4% trypan blue (Corning) and counted under a microscope. Hepa 1-6 murine liver cells (ATCC, #CRL-1830), used for gene expression analysis in the liver, and HEK293T cells (ATCC, #CRL-11268), used for transfections, were maintained in an identical fashion.

Gene expression analysis

RAW 264.7 murine macrophages were plated in 6-well plates at a density of 7.4 x 10^5 cells per well in DMEM containing 10% FBS and 1% PS/L-glut for 18 hours prior to treatment. After 18 hours, cells were treated with either vehicle control (DMSO), 200 μM DADS (Sigma, #2179-57-9), 900 μM AM (Sigma, #870-23-5), or 2.5 mM SAC (TCI, #21593-771; Portland, OR) for six hours. Doses and treatment times were determined by reviewing relevant literature and conducting preliminary trials (data not shown). As a positive control, cells were also treated with the pan-HDAC inhibitor Trichostatin A (TSA) (Millipore Sigma; #647926; Darmstadt, Germany) at 0.03 μM. Cells were treated for a total of six hours prior to extracting RNA for gene expression analyses or analysis of cell viability by the MTT assay.

*RNA Isolation, Reverse-Transcription and Gene Expression Analysis***.** At the end of the treatment period, total RNA was isolated using STAT-60 (Tel-Test, Inc.; Friendswood, TX), digested with DNase I (Roche Applied Science; Indianapolis, IN) and reverse-transcribed (Superscript II; Invitrogen; Carlsbad, CA). qPCR was performed using SYBR green (Applied Biosystems; Foster City, CA) chemistry on an ABI 7900HT. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method, with cyclophilin B as the invariant control.

Table 1: Primer sequences for qPCR.

Gene	**GenBank reference**	**Forward (5'-3')**	**Reverse (5'-3')**
mABCA1	NM_013454	CGTTTCCGGGAAGTGTCCTA	GCTAGAGATGACAAGGAGGATGGA
mCyclophilin	NM_011149	TGGAGAGCACCAAGACAGACA	TGCCGGAGTCGACAATGAT

mABCG1	NM_009593.1	GCTGTGCGTTTTGTGCTGTT	TGCAGCTCCAATCAGTAGTCCTAA
mHMGCR	BF464069	CTTGTGGAATGCCTTGTGATTG	AGCCGAAGCAGCACATGAT

Cell viability

MTT Assay. After 4 hours of treatment, MTT (Sigma) at 1 mg/mL final concentration was added to the media for an additional 2 hours. The media was removed and cells briefly washed, then treated with solubilization buffer (glycine). The absorbance of the plate was read at 570 nm. Due to a decrease in cell viability associated with SAC treatment, no further assays were completed using SAC.

Chromatin Immunoprecipitation*:*

ChIP was performed using a SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling, #9003; Danvers, MA) following the manufacturer's protocol. Because DADS is the most well-researched garlic-derived compound and most likely to function as an HDACi, ChIP was performed using only DADS and TSA as a positive control. Using the same dose (200 μM DADS or 0.03 μM TSA) and treatment time (six hours) as in gene expression analysis, $4x10^6$ cells per treatment group were cross-linked with 37% formaldehyde (VWR) for 10 minutes to preserve protein-DNA interactions. Cells were treated with micrococcal nuclease (included in kit) for 30 minutes to digest DNA to fragments of approximately 150-900 bp. Cells were then lysed using sonication (three sets of 20 second pulses on Fisher Scientific Sonic Dismembrator Model 100, level 5). Lysates were incubated with antibodies against acetylated histone H3 (Sigma, #06-599),

acetylated histone H4 (Sigma, #06-598), histone H3 (included in kit), and Rabbit IgG (included in kit). 10 μL of sample was set aside before antibodies were added to use as an input control. Samples were incubated overnight at 4°C with primary antibody.

After overnight incubation, Protein G Magnetic beads (included in kit) were added to each reaction and incubated to promote binding of the primary antibody to the beads. Using a magnetic rack (Cell Signaling), samples were washed to remove all unbound protein, and then washed again with an elution buffer to dissociate the DNA-protein fragments from the beads. Cross-links were reversed by adding NaCl and proteinase K (included in kit), and remaining DNA was purified using a spin column.

DNA was quantified using qPCR (ABI 7900HT) using SYBR green (Applied Biosystems). Primers were designed using Primer3 tool (Whitehead Institute for Biomedical Research; Cambridge, MA) coding for a series of regions up to 2500 base pairs upstream of the transcriptional start site of ABCA1 (**Figure 3**). This region contains the majority of known regulatory elements of ABCA1.

Table 2: Primer sequences for ChIP.

Region	Forward (5'-3')	Reverse (5'-3')
1	ACCGGTCAAACGCTGTTCTC	AGGGCCAGGGCTACAGAAAG
2	TGAGCCAGGGACTCCACATT	TTGGGGGCTGAGCAAACTAA
3	CCCAGATGCCAACCATTTTC	CCCTGGAAGGTGTGTGTGTG
4	TGGGTTTTGATGATGTCAAACG	AGGCTGGGTTCCATCTCTCC
5	TCGGAAGAAGAGTGCCAACA	TTTCCCCTTAGGCGTTTTCC
6	GGAAAACGCCTAAGGGGAAA	AGCAAGTGGATGCTGGGAAC
7	CCTCCATAAGCACCAGGTGTG	GCTTCTTTCTTCCCACCCTTTC
8	GCCAAATGCTTCATCCAGGT	TGGAAGGGAAAGCTCTCTGG
9	CCAGAGAGCTTTCCCTTCCA	GATCCCAGGGTCAGCTCAGA

Percent of input was calculated by $100 \times 2^{(\text{Input} - \text{Ct (IP)})}$.

Figure 3.

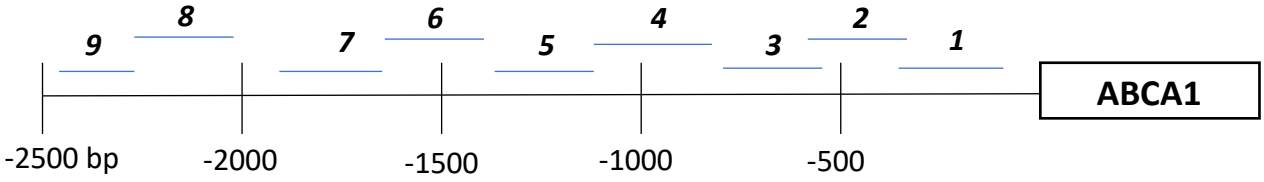


Figure 3: ABCA1 promoter mapping for chromatin immunoprecipitation (ChIP). qPCR primers (1-9) were designed using Primer3 tool [49] to cover almost the entire -2500 bp mouse ABCA1 promoter. Amplified fragments (blue) ranged from 178-216 bp in length.

Luciferase Assay

Plasmid Construction. Mouse ABCA1 promoter DNA was obtained from mouse liver genomic DNA (Zyagen; San Diego, CA) using PCR. The reverse primer amplified from -11 bp of the ABCA1 transcriptional start site (**Figure 5B**) and inserted an XhoI restriction site. This reverse primer was identical for all fragments. The forward primers inserted a BamHI restriction site and amplified regions from -76 to -2500 bp upstream of the ABCA1 transcriptional start site. PCR cycling conditions were as follows: 35 cycles at 94 °C for 15 seconds, 50 °C for 30 seconds, and 68°C for two minutes with Expand High-Fidelity PCR System (Roche). The PCR products were digested with BamHI and XhoI (New England Biolabs, Ipswich, MA). The luciferase vector was a TK-Luc plasmid [50] (**Figure 4**) similarly digested with BamHI and XhoI to remove TK promoter and linearize. The ABCA1 promoter fragments were then ligated into the luciferase plasmid (**Figure 5C**). The plasmid construct was confirmed by DNA sequencing (Oklahoma State University, DNA/Protein Core Facility; Stillwater, OK).

Transfection & Assay. Purified reporter plasmid constructs for 0.08, 0.25, 1.0, and 2.5 kilobases of the mouse ABCA1 promoter (0.1 μg) were transiently transfected into $2.1x10^4$ HEK293T cells (commonly used due to high propensity for transfections) using ViaFect transfection reagent (Promega, Madison, WI) at a 3:1 reagent:DNA ratio. Cells were cotransfected with 0.05 ng of the NanoLuc pNL-PGK vector (Promega). After 18 hours transfection time, cells were incubated with 200 μM DADS, 0.1 μM TSA, or vehicle (0.1% DMSO) in DMEM (10% FBS, no antibiotics) for six hours before assaying. Treatments were diluted in DMSO. Luciferase activity was detected using the Nano-Glo Dual-Luciferase Reporter Assay System (Promega, #N1610). Relative

Luminescence Units (RLUs) were determined by dividing luciferase luminescence by NanoLuc luminescence. To verify the assay, cells were separately transfected with a ΔTK-Luc plasmid containing 3 direct repeats of the LXR response element as the promoter and treated with LXR agonist T-compound (Sigma, #293754-55-9) at 1 μM for 18 hours. Luminescence was detected in the same fashion.

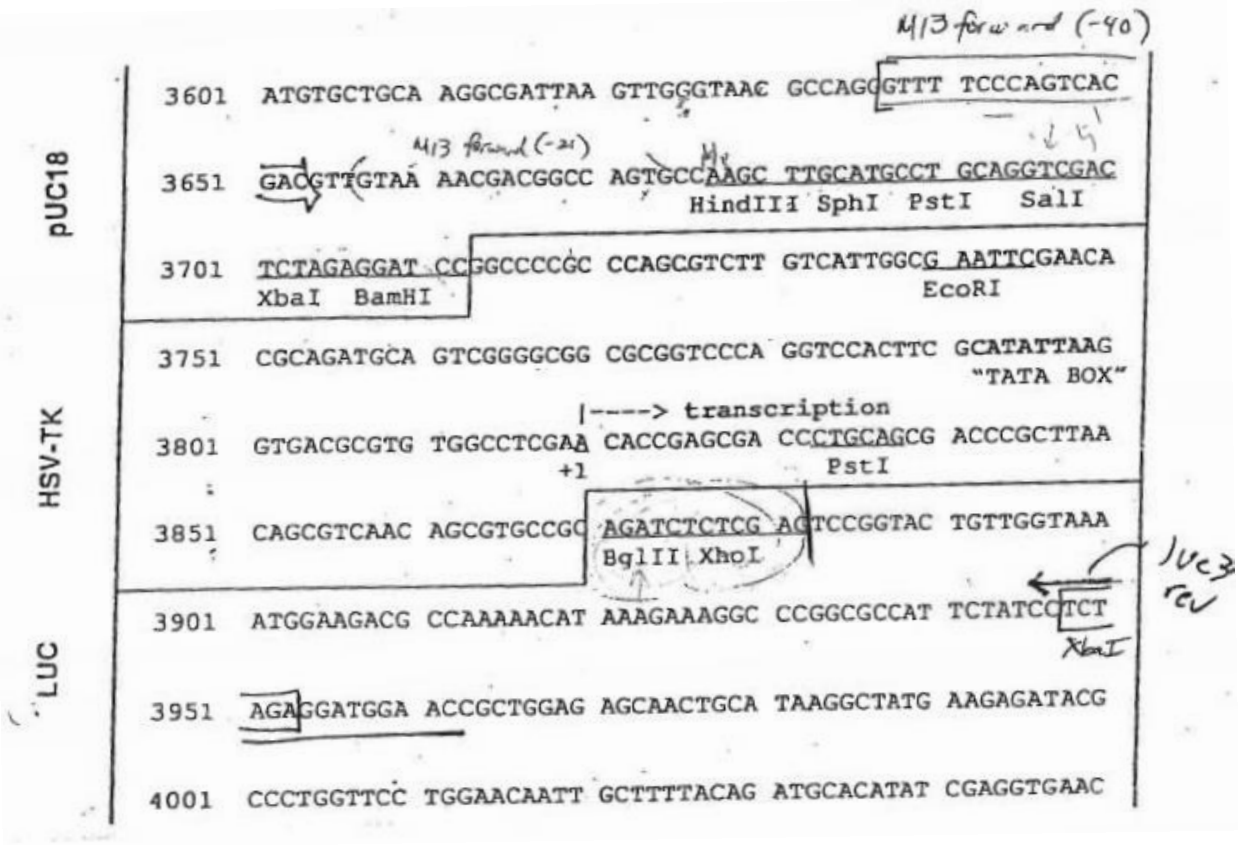
Statistical methods:

Statistical analyses were performed using a two-tailed *t*-test for assays involving only two groups. To compare differences between more than two groups, one-way ANOVA on SAS 9.4 software was used with a post hoc LSD procedure for pairwise comparisons. All data were expressed as mean ±SEM. Probability values of $p < 0.05$ were considered significant.

Figure 4.

Partial nucleotide sequence of plasmid tk-LUC (after S. Hollenberg)

HSV-TK promoter (-105 to +51, from pBLCAT2, Luckow & Schütz, (1987) NAR, 15, 5490) is placed between BamHI and BglII. Downsteram of XhoI, the firefly luciferase coding sequence with SV40 small t intron and poly-A signal (HIndIII - BamHI fragment from SVOA/L-AΔ5', de Wet et al., (1987) MCB, 7, 725-737) was cloned.



To clone any insert upstream of TK promoter, choose HindIII, SalI or BamHI. PstI cuts TK promoter, SphI cuts once in LUC seq., and XbaI cuts a margin between LUC and SV40 small t intron (releases Tk-LUC).

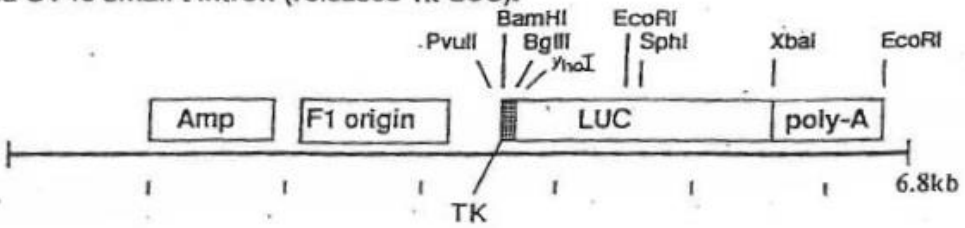
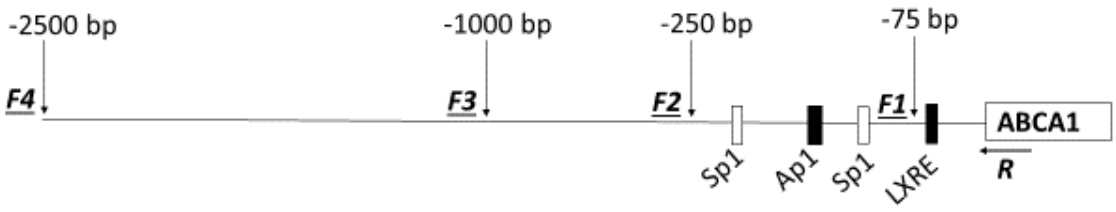


Figure 4: Map of TK-Luc plasmid used for ABCA1 promoter construct creation.
The shown plasmid, TK-Luc, contains a thymidine kinase (TK) promoter upstream of the luciferase gene. We used BamHI and XhoI to remove this promoter and inserted ABCA1 promoter fragments instead; thus, the finished plasmid is identical to this map aside from this promoter switch [50].

Figure 5.

A



B

Kb upstream of ABCA1 transcriptional start site	Primer sequence (5'-3')
0.08 (F1)	CAGCAGGGATCCACAGCGTTTGACCGGTAG
0.25 (F2)	CAGCAGGGATCCGGCCAGGGCTACAGAAAG
1.0 (F2)	CAGCAGGGATCCGGCAAGAGGATTAGGAAT
2.5 (F4)	CAGCAGGGATCCTTCTTGTATCTCTATGAC
Reverse (R)	CAGCAGGAGCTCTCGGAATTACTGGTTTTT

C

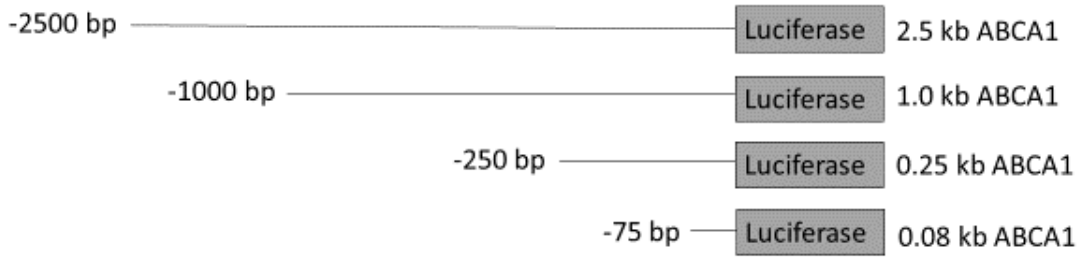


Figure 5: ABCA1 promoter regulatory elements and luciferase constructs. (A) The -2500 bp ABCA1 promoter has several binding motifs, including the Liver X Receptor Response Element (LXRE), multiple Sp1 binding sites, and an AP-1 binding site. (B) The entire mouse ABCA1 promoter was isolated from mouse genomic DNA using PCR. The reverse primer (R), identical for all fragments, amplified from -11bp of the ABCA1 transcriptional start site and engineered in an XhoI restriction site (underlined). The forward primers (F1-F4) amplified regions from -76 to -2500 bp upstream of the ABCA1 transcriptional start site, engineering in a BamHI restriction site (underlined). (C) The promoter fragments were inserted into a luciferase reporter vector.

CHAPTER IV

FINDINGS

Garlic-derived compounds DADS, AM, and SAC increase ABCA1 mRNA abundance

ABCA1 is the main cholesterol efflux transporter in macrophages, responsible for loading cholesterol onto high density lipoprotein (HDL). To determine the effects of garlic-derived compounds on mRNA expression of ABCA1, qPCR was performed on treated murine macrophage RAW 264.7 cells. Maximal effects on ABCA1 mRNA expression were observed at six hours (data not shown), so this time point was selected for all assays. Analysis revealed an eight-fold increase in mRNA due to treatment with DADS, and around a four-fold increase for AM and SAC (**Figure 6**). Pan-HDAC inhibitor TSA also caused a four-fold increase in ABCA1 mRNA.

At the determined doses of 200 μM DADS, 900 μM AM, 2.5 mM SAC, and 0.01 μM TSA, MTT assay for cell viability showed a significantly lower cell viability in SAC-treated cells after six hours (**Figure 7**). Due to this effect on SAC-treated cells, we did not carry out further gene expression analysis for this compound.

The LXR pathway is known to activate not only ABCA1, but also genes such as ATP-binding cassette transporter G1 (ABCG1), which encodes a secondary cholesterol efflux vehicle [17]. Additionally, AM specifically has been shown to increase cholesterol synthesis in human liver cells [36], and HMG Co-A Reductase (HMGCR) is the rate-controlling enzyme of the mevalonate pathway that produces cholesterol. Treatment of RAW 264.7 cells with DADS, AM, or TSA showed no significant differences in ABCG1 (**Figure 8A**), and murine liver Hepa 1-6 cells treated with AM showed no significant differences in expression of HMGCR (**Figure 8B**).

DADS does not increase levels of acetylated histone H3 or H4 in the ABCA1 promoter

The present study used chromatin immunoprecipitation assays to examine histone modification in nine regions of the 2500 bp ABCA1 promoter (**Figure 3**). We found no significant differences in acetylated histone H3 (**Figure 9A**) or H4 (**9B**) in any region of the 2500 bp ABCA1 promoter related to treatment with DADS or pan-HDAC inhibitor TSA. Although TSA appeared to increase levels of histone H4 in almost every segment amplified by qPCR, this trend was not statistically significant. Measures of positive control histone H3 (unmodified and equally present in all chromatin) (**Figure 9C**) and negative control IgG (**9D**) were not significantly different based on treatment with either DADS or TSA.

DADS increases ABCA1 promoter luciferase activity

In order to determine whether the effect of DADS on putative ABCA1 promoter activation would persist in a histone-independent state and whether activation was

specific to a certain region of the promoter, four different promoter-fragment/luciferase plasmid constructs were created with progressively longer segments of the promoter. These circularized plasmids were transiently expressed in HEK293T cells, where histones exist naturally but are not associated with the plasmids.

To verify the assay, a plasmid containing a direct repeat of the LXRE preceding the luciferase gene was transfected and treated with LXR agonist T-compound. When this plasmid was cotransfected with LXRα/RXRα (required heterodimer for LXR activation), T-compound treatment caused significantly increased luciferase activity (**Figure 10**). LXRα/RXRα is normally present in HEK293T cells, but cotransfection to increase expression was required in this short promoter construct to see increases in transcription with T-compound treatment. In further assays using longer ABCA1 promoter constructs (all but the 0.08 kb construct), T-compound promoter activation was seen independent of LXRα/RXRα cotransfection (data not shown).

In HEK293T cells transfected with the ABCA1-luciferase plasmids, basal promoter activity was increased as promoter size increased (**Figure 11**). In all promoter constructs, six hour treatment with DADS significantly increased luciferase activity (**Figure 12**). Treatment with TSA caused a similar increase.

Figure 6.

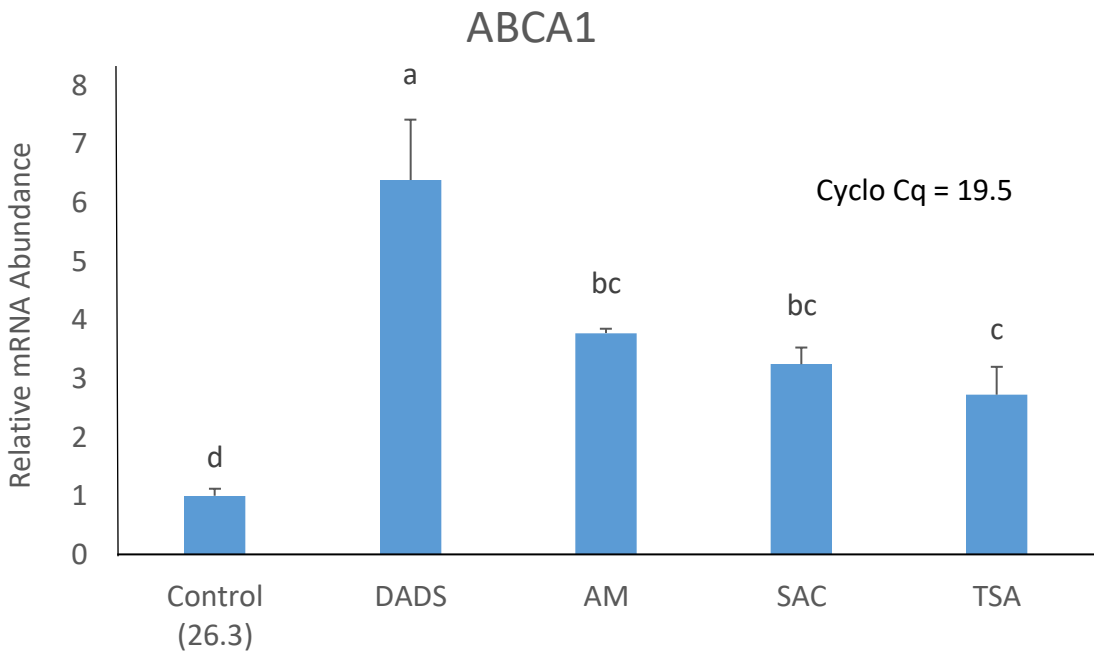


Figure 6: Effect of six hour garlic compound treatment on ABCA1 mRNA expression in RAW 264.7 cells. Cells seeded at $3.7x10^5$ cells/mL were treated with 0.1% DMSO (vehicle control), 200 μM DADS, 900 μM AM, 2.5 mM SAC, or 0.01 μM TSA for six hours. Total RNA was isolated and reverse transcribed, and ABCA1 expression was evaluated by real-time polymerase chain reaction (qPCR). Analysis was done using the $2^{-\Delta\Delta Ct}$ method, with cyclophilin B as the invariant control. Significance was calculated using a one-way ANOVA on SAS software with $\alpha=0.05$ and a post hoc LSD test. Groups that lack a common letter are significantly different.

Figure 7.

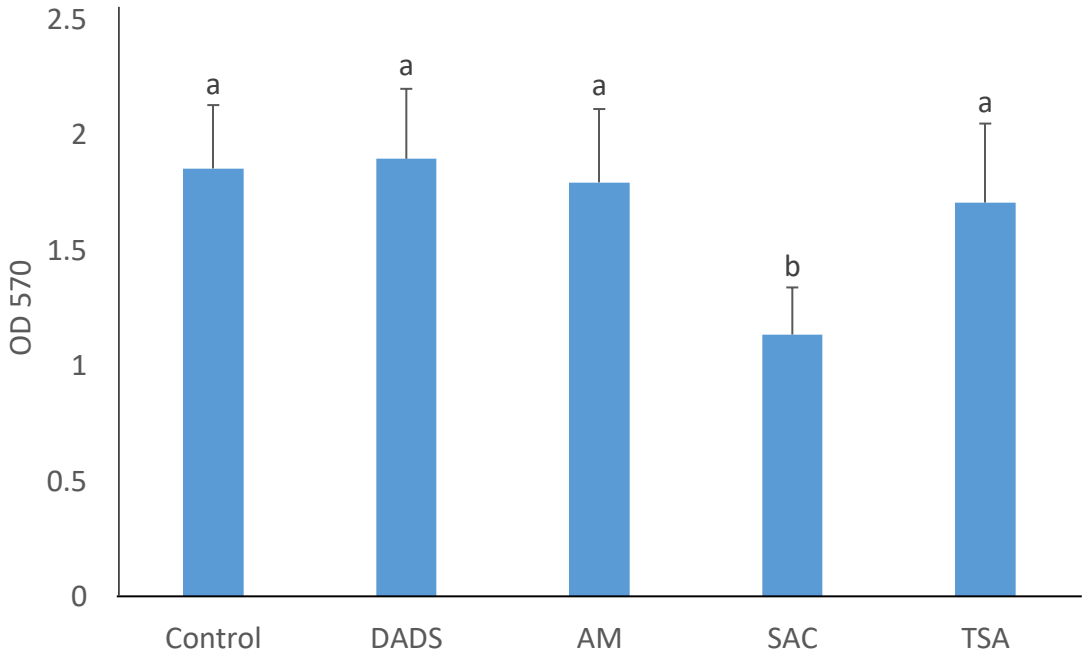
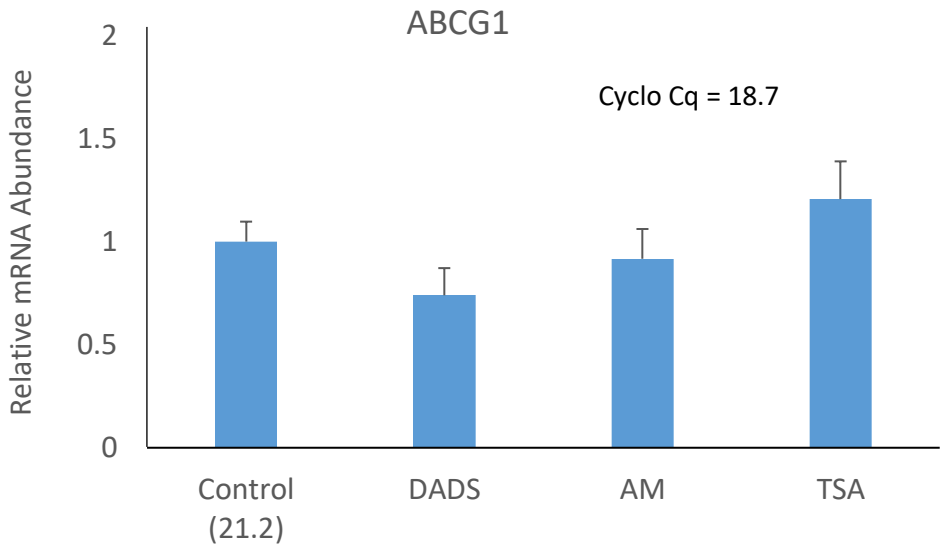


Figure 7: Effect of six hour garlic compound treatment on RAW 264.7 cell viability as determined by MTT assay. Cells seeded at $3.7x10^5$ cells/mL were treated with 0.1% DMSO (vehicle control), 200 μM DADS, 900 μM AM, 2.5 mM SAC, or 0.01 μM TSA for four hours, after which 5 mg/ml MTT was added for an additional two hours (six hours total treatment length). The plate was read at OD 570. Significance was calculated using a one-way ANOVA on SAS software with $\alpha=0.05$, and post hoc LSD procedure for pairwise comparison. Groups that lack a common letter are significantly different.

Figure 8.

A


ABCG1
Cyclo Cq = 18.7
Relative mRNA Abundance
2
1.5
1
0.5
0
Control
(21.2)
DADS
AM
TSA

B

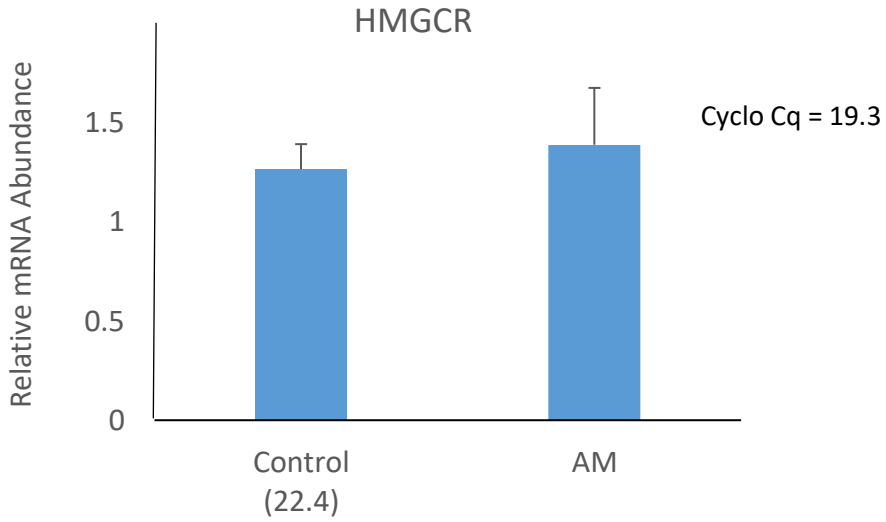
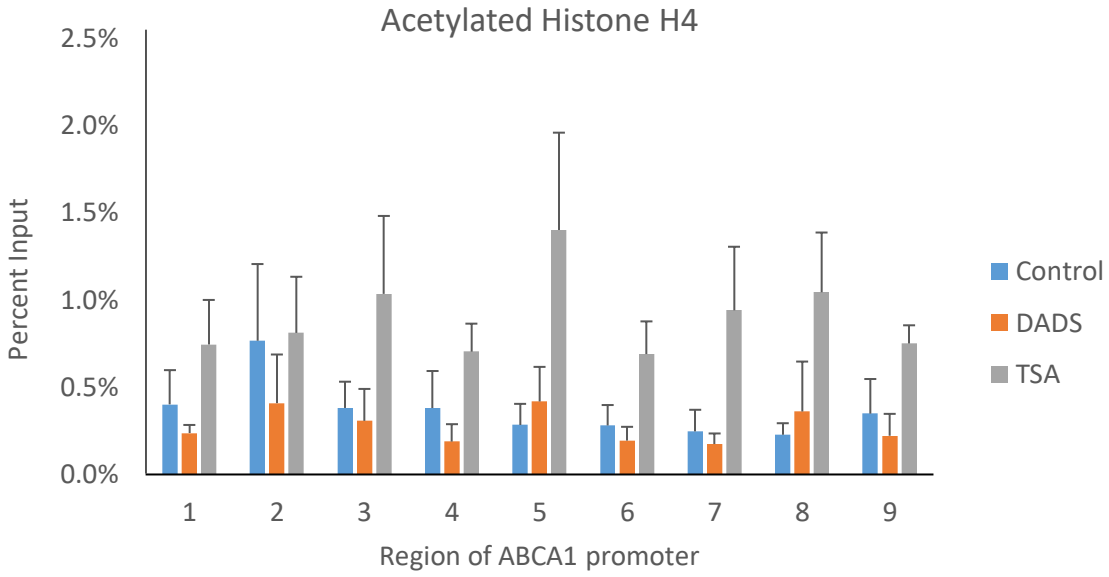

HMGCR
Cyclo Cq = 19.3
Relative mRNA Abundance
1.5
1
0.5
0
Control
(22.4)
AM

Figure 8: Effects of garlic compounds on ABCG1 and HMGCR. (**A**) RAW 264.7 cells seeded at $3.7x10^5$ cells/mL were treated with 0.1% DMSO, 200 μM DADS, 900 μM AM, or 0.01 μM TSA for six hours. (**B**) Hepa 1-6 cells seeded at $3.7x10^5$ cells/mL were treated with 0.1% DMSO or 900 μM AM for six hours.

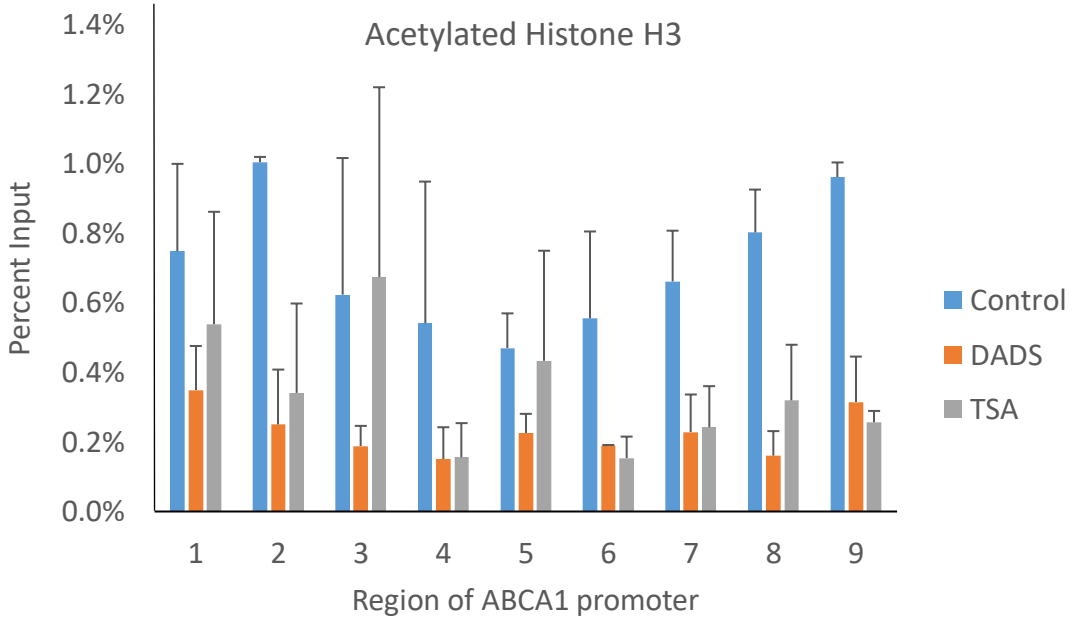
After treatment, total RNA for each group was isolated and reverse transcribed, and gene expression was evaluated by quantitative real-time polymerase chain reaction (qPCR). RAW 264.7 cells (**A**) were analyzed for expression of ABCA1; Hepa 1-6 cells (**B**) were analyzed for expression of HMGCR. Analysis was done using the $2^{-\Delta\Delta Ct}$ method, with cyclophilin B as the invariant control. Significance was calculated using a one-way ANOVA on SAS software and post hoc LSD procedure for pairwise comparison (**A**) or a *t*-test (**B**) with $\alpha=0.05$.

Figure 9.

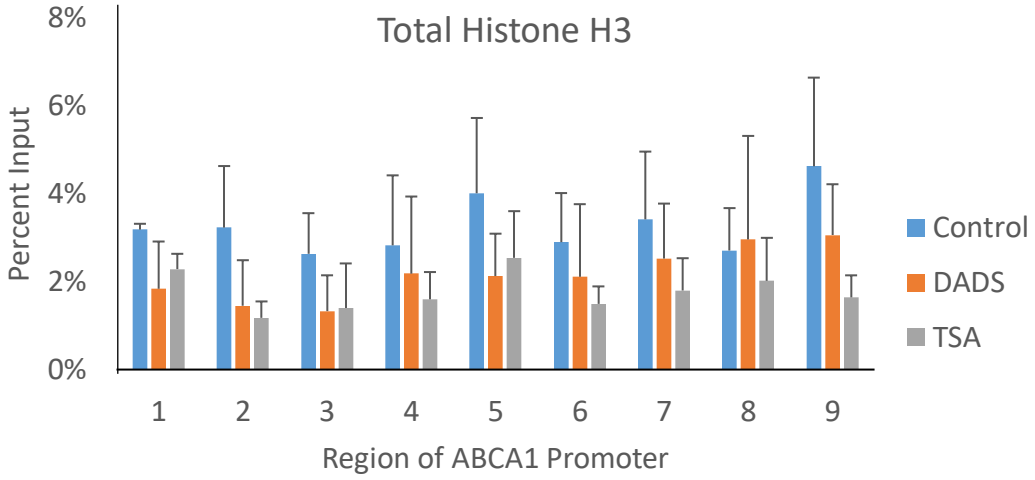
A


Acetylated Histone H4
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
Percent Input
1
2
3
4
5
6
7
8
9
Region of ABCA1 promoter
Control
DADS
TSA

B


Acetylated Histone H3
1.4%
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
Percent Input
1
2
3
4
5
6
7
8
9
Region of ABCA1 promoter
Control
DADS
TSA

C


Total Histone H3
8%
6%
4%
2%
0%
Percent Input
1
2
3
4
5
6
7
8
9
Region of ABCA1 Promoter
Control
DADS
TSA

D

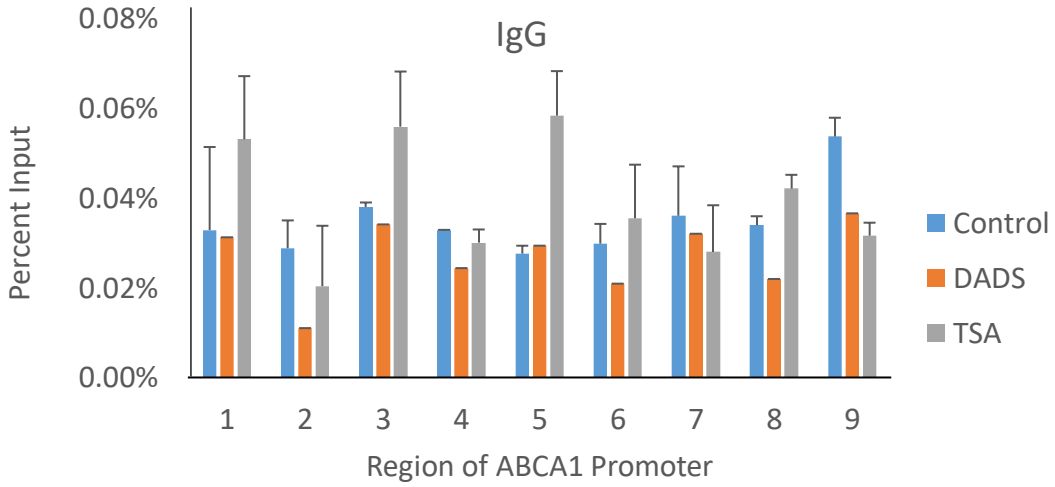

IgG
0.08%
0.06%
0.04%
0.02%
0.00%
Percent Input
1
2
3
4
5
6
7
8
9
Region of ABCA1 Promoter
Control
DADS
TSA

Figure 9: Effect of DADS treatment on amount of acetylated histone H3 and H4 in mouse ABCA1 promoter in RAW 264.7 cells. After six hour treatment with 200 μM DADS or 0.01 μM TSA, $4x10^6$ RAW 264.7 cells per treatment group were cross-linked with formaldehyde and digested with nuclease. Cells were lysed using sonication, and lysates incubated with antibodies. After washing and reversing cross-links, purified DNA was quantified using qPCR (primers shown in **Table 2**). Percent of input was calculated by $100 \times 2^{(\text{Input} - \text{Ct (IP)})}$.

Figure displays results for acetylated histone H4 (**A**); acetylated histone H3 (**B**); total histone H3 (positive control) (**C**); and IgG (negative control) (**D**).

Significance was calculated using one-way ANOVA with $\alpha=0.05$. No significant differences based on treatment were found in any region of the promoter for any antibody used.

Figure 10.

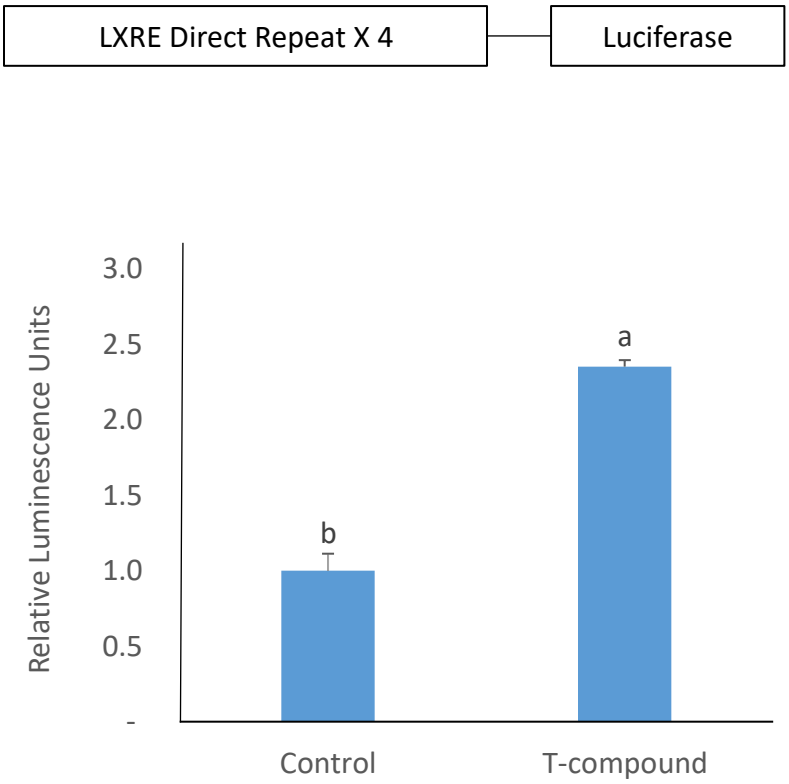


Figure 10: Effect of T-compound on DR4X3 plasmid. 0.07 µg DR4X3 plasmid was transfected into $2.1x10^4$ HEK293T cells in the presence of RXRα (0.03 µg) and LXRα (0.03 µg). Cells were cotransfected with 0.5 ng NanoLuc pNL-PGK vector to normalize differences in transfection efficiency. Six hours after transfection, cells were treated with 1 µM T-compound for 18 hours. Luciferase activity was detected as relative luminescence units (RLUs) using the Nano-Glo Dual-Luciferase Reporter Assay System. Significance was calculated using a two-tailed *t*-test with α=0.05. Groups that lack a common letter are significantly different.

Figure 11.

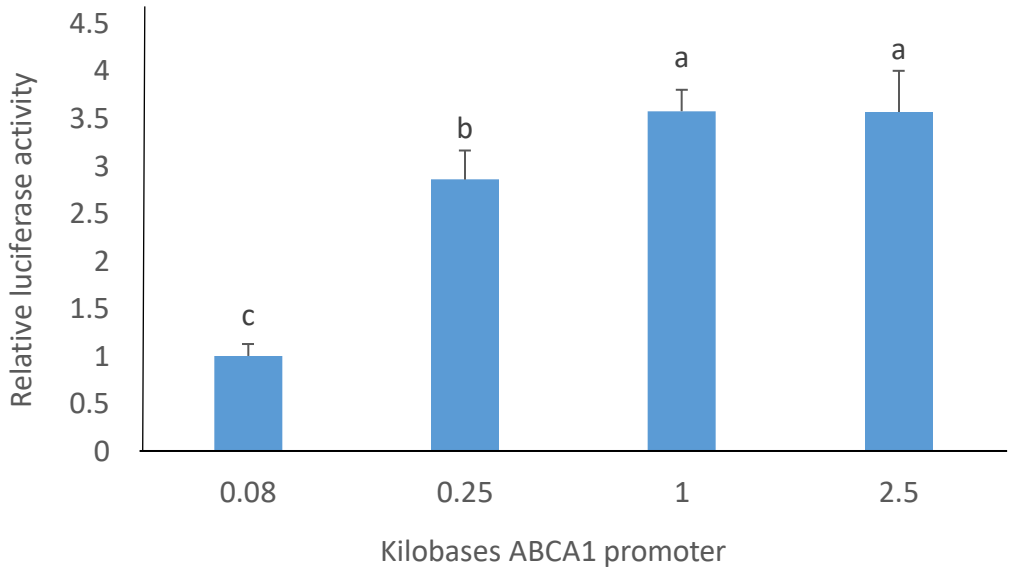


Figure 11: Differing levels of basal luciferase activity based on plasmid size. Purified reporter plasmid constructs for 0.08, 0.25, 1.0, and 2.5 kilobases of the mouse ABCA1 promoter (0.1 µg) were transiently transfected into $2.1x10^4$ HEK293T cells. Cells were cotransfected with 0.05 ng of the NanoLuc pNL-PGK vector. After 24 hours, luciferase activity was detected using the Nano-Glo Dual-Luciferase Reporter Assay System. Relative luciferase activity was determined by dividing luciferase luminescence by NanoLuc luminescence. Significance was calculated using one-way ANOVA analysis on SAS software with $\alpha=0.05$, and post hoc LSD procedure for pairwise comparison. Groups that lack a common letter are significantly different.

Figure 12.

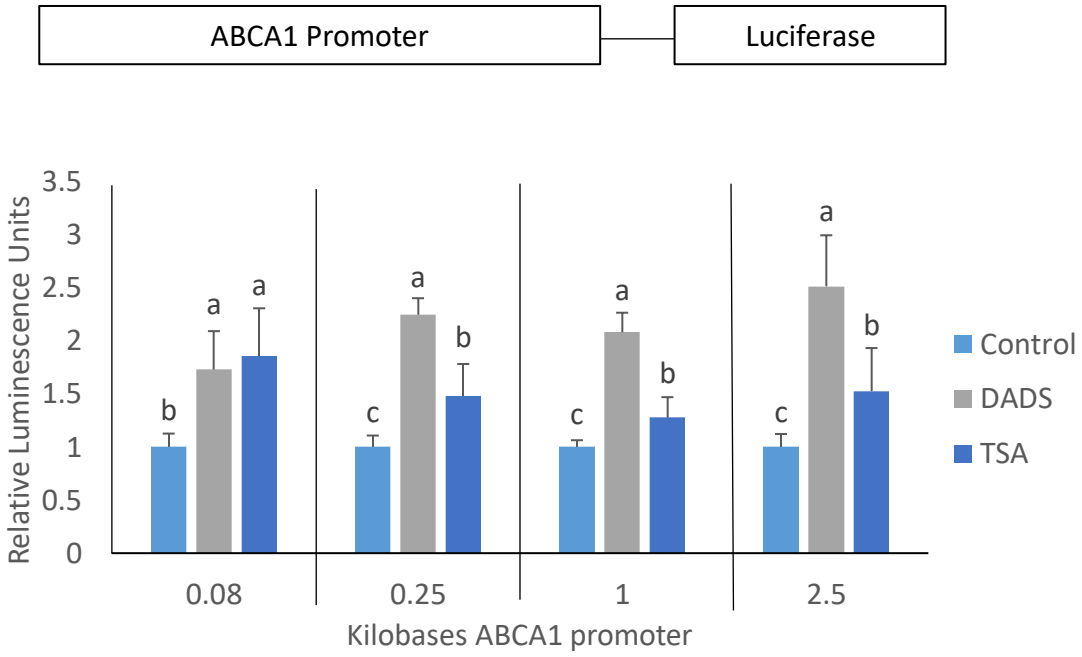


Figure 12: Effect of DADS and TSA on ABCA1 promoter-luciferase plasmids. Purified reporter plasmid constructs for 0.08, 0.25, 1.0, and 2.5 kilobases of the mouse ABCA1 promoter (0.1 µg) were transiently transfected into $2.1x10^4$ HEK293T cells. Cells were cotransfected with 0.05 ng of the NanoLuc pNL-PGK vector. After 18 hours transfection time, cells were incubated with 200 µM DADS, 0.1 µM TSA, or vehicle (0.1% DMSO) in DMEM (10% FBS) for six hours before assaying. Luciferase activity was detected using the Nano-Glo Dual-Luciferase Reporter Assay System. Relative Luminescence Units (RLUs) were determined by dividing luciferase luminescence by NanoLuc luminescence. Significance was calculated using one-way ANOVA analysis on SAS software with α=0.05, and a LSD post hoc procedure for pairwise comparison within groups. Groups that lack a common letter (within a given plasmid construct) are significantly different.

CHAPTER V

CONCLUSION

Changes in mRNA expression due to garlic compound treatment

In this study, treatment with garlic-derived compounds DADS, AM, and SAC increased ABCA1 mRNA expression significantly in RAW 264.7 murine macrophage cells. The importance of the role of ABCA1 in cholesterol efflux is highlighted in Tangier disease, where a mutation of ABCA1 causes severe cholesterol buildup, hepatosplenomegaly, peripheral neuropathy, and increased risk of CVD [13]. Conversely, increasing expression of ABCA1 has been linked to an inhibition of atherosclerotic lesion progression due to increased reverse cholesterol transport [14]. According to Wang et al. [51] increased ABCA1 expression in macrophages improved HDL-c levels, and may improve cholesterol efflux to HDL to slow or delay the development of atherosclerosis. Due to the potentially antiatherogenic effects of increasing this cholesterol transporter, the upregulation of ABCA1 mRNA expression by garlic compounds DADS, AM, and SAC may be protective against atherosclerosis. Associations of garlic supplementation with reduced serum cholesterol in human studies support this idea.

ABCG1 is a secondary cholesterol efflux vehicle present in macrophages. In the present study, treatment with DADS, AM, and TSA failed to increase ABCG1 expression. This suggests that the increase in ABCA1 expression may not be mediated by the LXR,

because LXR activation leads to an increase of ABCG1 [17].

Some studies suggest that garlic works to reduce serum cholesterol levels by decreasing cholesterol synthesis. In human Hep-G2 cells, AM (0.3 mM-1.3 mM) significantly inhibited ^{3}H-acetate incorporation into cholesterol (up to 80%) as well as cholesterol secretion into medium (up to 50%) after a four hour treatment period [36]. In the present study in murine Hepa 1-6 liver cells, AM did not cause a significantly higher mRNA expression of HMG Co-A Reductase, the rate-limiting enzyme in the cholesterol synthesis pathway and the main target of cholesterol-reducing medications such as atorvastatin (Lipitor) and fluvastatin (Lesecol) [52, 53]. The effects of AM on cholesterol synthesis may not be mediated by increasing mRNA transcription of this enzyme; HMGCR activity was shown to be reduced in rat hepatocytes treated with allicin and ajoene (another byproduct of allicin degradation) [54], and cholesterol synthesis can be regulated by post-transcriptional modification of HMGCR [55]. Treatment of Hepa 1-6 cells with DADS and AM did not result in significantly higher mRNA expression of ABCA1 (data not shown).

Garlic compound effect on cell viability

In previous studies, garlic compounds have been shown to be cytotoxic or to inhibit cell proliferation at high levels. Liao et al [56] found DADS to significantly inhibit cell proliferation in human colon cancer SW480 cells at levels from 205-480 μM. In the previously mentioned study by Cho et al. testing AM treatment effect on cholesterol synthesis, AM caused decreased cell viability at levels above 3.4 mM, although the lower levels of 0.3-1.3 mM that reduced cholesterol synthesis and secretion

were not found to be significantly detrimental [36]. In the present study at doses of 200 μM DADS, 900 μM AM, and 2.5 mM SAC (doses determined by literature and preliminary trials), MTT assay showed significantly lower viability in SAC-treated RAW 264.7 cells. Although SAC is thought to be up to 30 times less toxic than DADS and allicin [57], the levels needed to cause a significant increase in ABCA1 mRNA (2.5 mM) were cytotoxic in this study; SAC did not affect ABCA1 mRNA levels at doses below 2.5 mM (data not shown). Due to this cytotoxic effect, we did not continue further testing with SAC.

Effect of DADS on histone acetylation

Studies show that DADS specifically may work as a histone deacetylase inhibitor. Druesne et al [48] found that in human colon cancer Caco-2 and HT-29 cells, treatment with 200 μM DADS for six hours significantly increased acetylated histone in the p21 promoter, leading to increased transcription and translation. Histone acetylation was evaluated by histone extraction and western blotting, and DADS increased acetylation of histone H3 in both cell lines and H4 only in Caco-2. A follow-up study using chromatin immunoprecipitation in conjunction with PCR confirmed this finding. Zhao et al. [58] found that expression of acetylated histone H3 and H4 was significantly increased in human acute myeloid leukemia HL-60 cells, and this expression was linked to an increase in p21 protein.

Chromatin immunoprecipitation assays in the present study indicated no significant differences in acetylated histone H3 or H4 in any region of the 2500 bp ABCA1 promoter related to treatment with DADS or pan-HDAC inhibitor TSA, despite

increases in ABCA1 mRNA abundance. The antibodies used to capture acetylated histone H3 and H4 were both pan-histone antibodies, meaning they were nonspecific to certain lysine residues. Acetylation of certain residues (Ac-H3K9 and Ac-H3K18) has specifically been linked to transcriptional upregulation [59, 60], while the acetylation of other residues may have other functions such as affecting methylation and DNA repair [61]. If the transcriptional upregulation of ABCA1 due to garlic treatment is due to acetylation at only one specific lysine site, the effect may not have been large enough to have been captured by the pan-antibody.

Additionally, due to the lack of current research about the specific area of the ABCA1 promoter targeted by garlic compounds, the primers we designed covered most portions of the 2,500 bp promoter, but not all of it (50-100 bp gaps between primer fragments). If an unknown transcription binding motif exists in a region not covered by our primers, the effect would not be shown in our results. All of these are possible explanations for the lack of increase in acetylated histone due to garlic treatment.

Although TSA appeared to increase levels of histone H4 in almost every segment amplified by qPCR, this trend was not statistically significant.

DADS increases ABCA1 promoter transcription in transiently transfected plasmids

DADS (and TSA) significantly increased luciferase activity of all ABCA1 promoter fragments used regardless of length. Although DADS may be working as an HDAC to increase gene expression, transiently transfected plasmids are not associated with the chromatin structure. Thus, transcriptional upregulation may not be a direct result of histone modification in the ABCA1 promoter and there must be another mechanism to

explain these results. Bao et al. found that TSA works in the promoter of SR-B1, another cholesterol efflux transporter, by binding to the transcription element Sp1 and/or SRE [62]. In that study when luciferase reporter constructs were created using variations of the SR-B1 promoter, luciferase activity was significantly higher in segments containing the Sp1 and SRE elements than in segments not including these binding regions [62].

In contrast, all ABCA1 promoter fragments in the current study treated with DADS had elevated luciferase activity, even as close to the transcriptional start site as 75 bp. TSA had the same effect. It is possible that both DADS and TSA activate the LXRE, which is included in the 0.08 KB ABCA1-Luciferase construct; however, no studies to date have demonstrated a role for either of these compounds in activating the LXRE, and other target genes of LXR such as ABCG1 and HMGCR [17] were not affected by treatment with DADS, AM, or TSA in qPCR analysis of mRNA abundance.

Another more viable explanation for this increase in luciferase activity is that both DADS and TSA upregulate transcription independent of a specific binding element (i.e. increase basal transcription rates). In a similar luciferase reporter construct, Ashburner et al. found that inhibition of HDAC activity via TSA treatment increased NF-κB-dependent gene expression, whereas HDAC1 and HDAC2 repressed gene expression [63].

Recently, over 50 non-histone proteins have emerged as substrates for HDAC. This group includes transcription factors such as p53, nuclear factor kB (NF-kB), hypoxia-inducible factor 1 alpha (HIF-1α), E2F1, and RUNX3 [64]. TSA was shown to stabilize acetylation of transcription factor RUNX3 by inhibiting HDAC that normally target RUNX3 for degradation through deacetylation [65]. It is possible that DADS

and/or TSA work as HDACi to modify either non-histone proteins or histones not found in the chromatin scaffolding of the ABCA1 promoter that then cause a transcriptional upregulation of ABCA1.

Limitations

There are several limitations to the current study. The cell culture model of study allows for great manipulation of variables such as dose and time of treatment, and the conclusions of cell culture studies are used to design improved future trials in animals and humans. However, it can be difficult to directly translate these results into conclusions for tissues and whole organisms. Increases in ABCA1 and/or cholesterol efflux in vitro may not directly correlate with similar increases in an animal or human.

Assuming the increase in ABCA1 mRNA is due to a transcriptional upregulation is not necessarily the only conclusion; an increase in mRNA stability is another possible explanation for the increase seen in qPCR. We did not explore this possibility in the current study.

The ChIP assay was limited by antibody specificity. The pan-acetyl-H3 and pan-acetyl-H4 antibodies were meant to capture a wide range of histone modifications, but the lack of significant differences due to DADS treatment may be due to a specific modification not captured by the antibodies.

In the luciferase assay, both DADS and TSA increased transcription of all ABCA1 promoter fragments, and T-compound increased transcription of all but the shortest. Vehicle control (DMSO) was used as the baseline; however, no negative control was incorporated. Creating another plasmid construct with a gene such as ABCG1 known

to not be transcriptionally upregulated by DADS would be helpful in determining whether these results are specific to the ABCA1 promoter.

In conclusion, the presented results demonstrate that garlic compounds DADS, AM, and SAC increase mRNA expression of ABCA1 in RAW 264.7 macrophages, and this effect may be due to modification of histone proteins not directly associated with the ABCA1 promoter chromatin. Future research should involve western blot assay for ABCA1 protein to confirm that the increase in mRNA leads to an increase in protein, cholesterol efflux assays to confirm that garlic treatment functionally increases efflux, and chromatin immunoprecipitation assays using more specific antibodies such as H3K9 and H3K18 [66]. Luciferase assays should be repeated using constructs for a gene such as ABCG1 that should not be transcriptionally upregulated by DADS.

REFERENCES

[1] Mozaffarian, D., et al. (2016). "Heart Disease and Stroke Statistics—2016 Update." A Report From the American Heart Association 133(4): e38-e360.

[2] Oklahoma, S. o. (2016). Heart Disease in Oklahoma, Oklahoma State Department of Health; Chronic Disease Service.

[3] Falk, E. (2006). "Pathogenesis of Atherosclerosis." Journal of the American College of Cardiology 47(8): C7-C12.

[4] Libby, P., et al. (2011). "Progress and challenges in translating the biology of atherosclerosis." Nature 473: 317.

[5] Grundy, S. M., et al. (2004). "Implications of Recent Clinical Trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines." Circulation 110(2): 227-239.

[6] Yamamoto, S., et al. (2016). "The macrophage and its related cholesterol efflux as a HDL function index in atherosclerosis." Clinica Chimica Acta 457: 117-122.

[7] Law, M. R., et al. (1994). "By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischaemic heart disease?" Bmj 308(6925): 367.

[8] Rader, D. J. (2006). "Molecular regulation of HDL metabolism and function: implications for novel therapies." The Journal of clinical investigation 116(12): 3090.

[9] Miller, G. J. and N. E. Miller (1975). "Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease." Lancet (London, England) 1(7897): 16.

[10] Rohatgi, A., et al. (2014). "HDL Cholesterol Efflux Capacity and Incident Cardiovascular Events." The New England Journal of Medicine 371(25): 2383-2393.

[11] Singaraja, R. R., et al. (2003). "Efflux and atherosclerosis: the clinical and biochemical impact of variations in the ABCA1 gene." Arterioscler Thromb Vasc Biol 23(8): 1322-1332.

[12] Chiang, J. Y. L. (2004). "Regulation of bile acid synthesis: pathways, nuclear receptors, and mechanisms." Journal of Hepatology 40(3): 539-551.

[13] Angela, B.-W., et al. (1999). "Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency." Nature Genetics 22(4): 336.

[14] Van Eck, M., et al. (2006). "Macrophage ATP-Binding Cassette Transporter 1 Overexpression Inhibits Atherosclerotic Lesion Progression in Low-Density Lipoprotein Receptor Knockout Mice." Arteriosclerosis, Thrombosis, and Vascular Biology.

[15] Oram, J. F., et al. (2000). "ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages." The Journal of biological chemistry 275(44): 34508.

[16] Chen, X., et al. (2011). "Transcriptional regulation of ATP-binding cassette transporter A1 expression by a novel signaling pathway." J Biol Chem 286(11): 8917-8923.

[17] Kalaany, N. Y. and D. J. Mangelsdorf (2006). "LXRS and FXR: the yin and yang of cholesterol and fat metabolism." Annu Rev Physiol 68: 159-191.

[18] O'Connor, L., et al. (2016). "The Role of the Ubiquitously Expressed Transcription Factor Sp1 in Tissue-specific Transcriptional Regulation and in Disease." The Yale Journal of Biology and Medicine 89(4): 513-525.

[19] Trop-Steinberg, S. and Y. Azar (2017). "AP-1 Expression and its Clinical Relevance in Immune Disorders and Cancer." The American Journal of the Medical Sciences 353(5): 474-483.

[20] Santamarina-Fojo, S., et al. (2000). "Complete genomic sequence of the human ABCA1 gene: analysis of the human and mouse ATP-binding cassette A promoter." Proc Natl Acad Sci U S A 97(14): 7987-7992.

[21] Schreiber, D. H. and T. R. Anderson (2006). "Statin-induced rhabdomyolysis." Journal of Emergency Medicine 31(2): 177-180.

[22] Sattar, N. and M.-R. Taskinen (2012). "Statins are diabetogenic – Myth or reality?" Atherosclerosis (Supplements) (Component) 13(1): 1-10.

[23] Yu, E., et al. (2016). "Diet, Lifestyle, Biomarkers, Genetic Factors, and Risk of Cardiovascular Disease in the Nurses' Health Studies." American journal of public health 106(9): 1616.

[24] Heller, M. (2016). "What Is the DASH Diet?". Retrieved January 31, 2017, 2017, from http://dashdiet.org/what_is_the_dash_diet.asp.

[25] Salehi-Abargouei, A., et al. (2013). "Effects of Dietary Approaches to Stop Hypertension (DASH)-style diet on fatal or nonfatal cardiovascular diseases—Incidence: A systematic review and meta-analysis on observational prospective studies." Nutrition 29(4): 611-618

[26] Rahman, K. and G. Lowe (2006). "Garlic and Cardiovascular Disease: A Critical Review1,2." The Journal of Nutrition 136(3S): 736S-740S.

[27] Amagase, H. (2006). "Clarifying the real bioactive constituents of garlic." J Nutr 136(3 Suppl): 716s-725s.

[28] Freeman, F. and Y. Kodera (1995). "Garlic Chemistry: Stability of S-(2-Propenyl)-2-Propene-1-sulfinothioate (Allicin) in Blood, Solvents, and Simulated Physiological Fluids." Journal of Agricultural and Food Chemistry 43(9): 2332-2338.

[29] Brodnitz, M. H., et al. (1971). "Flavor components of garlic extract." Ibid. 19(2): 273-275.

[30] Sheen, L. Y., et al. (1999). "Metabolites of Diallyl Disulfide and Diallyl Sulfide in Primary Rat Hepatocytes." Food and Chemical Toxicology 37(12): 1139-1146.

[31] Minami, T., et al. (1989). "Odor Components of Human Breath After the Ingestion of Grated Raw Garlic." Journal of Food Science 54(3): 763-763

[32] Yoshimoto, N., et al. (2015). "Garlic γ-glutamyl transpeptidases that catalyze deglutamylation of biosynthetic intermediate of alliin." Frontiers in Plant Science 5(758).

[33] Banerjee, S. K. and S. K. Maulik (2002). "Effect of garlic on cardiovascular disorders: a review." Nutrition Journal 1: 4-4.

[34] Jandke, J. and G. Spiteller (1987). "Unusual conjugates in biological profiles originating from consumption of onions and garlic." J Chromatogr 421(1): 1-8.

[35] Ried, K., et al. (2013). "Effect of garlic on serum lipids: an updated meta-analysis." Nutrition Reviews 71(5): 282-299.

[36] Cho, B. H. and S. Xu (2000). "Effects of allyl mercaptan and various allium-derived compounds on cholesterol synthesis and secretion in Hep-G2 cells." Comp Biochem Physiol C Toxicol Pharmacol 126(2): 195-201.

[37] Lee, S., et al. (2012). "High hydrostatic pressure extract of garlic increases the HDL cholesterol level via up-regulation of apolipoprotein A-I gene expression in rats fed a high-fat diet." Lipids in health and disease 11: 77.

[38] Malekpour-Dehkordi, Z., et al. (2013). "S-Allylcysteine, a Garlic Compound, Increases ABCA1 Expression in Human THP-1 Macrophages." Phytotherapy Research 27(3): 357-361.

[39] Grunstein, M. (1997). "Histone acetylation in chromatin structure and transcription." Nature 389(6649): 349-352.

[40] Inkster, B., et al. (2013). "Histone deacetylase gene variants predict brain volume changes in multiple sclerosis." Neurobiol Aging 34(1): 238-247.

[41] Keir, J. M., et al. (2015). "Protein acetylation in metabolism—metabolites and cofactors." Nature Reviews Endocrinology.

[42] Song, Y., et al. (2011). "The significance of strong histone deacetylase 1 expression in the progression of prostate cancer." Histopathology 58(5): 773-780.

[43] Ozdag, H., et al. (2006). "Differential expression of selected histone modifier genes in human solid cancers." BMC Genomics 7: 90.

[44] Chen, X., et al. (2012). "Requirement for the histone deacetylase Hdac3 for the inflammatory gene expression program in macrophages." Proc Natl Acad Sci U S A 109(42): E2865-2874.

[45] Shakespear, M. R., et al. (2013). "Histone deacetylase 7 promotes Toll-like receptor 4-dependent proinflammatory gene expression in macrophages." J Biol Chem 288(35): 25362-25374.

[46] Shalome, A. B. and P. G. B. Matthew (2014). "The Role of Dietary Histone Deacetylases (HDACs) Inhibitors in Health and Disease." Nutrients 6(10): 4273-4301.

[47] Nian, H., et al. (2008). "Allyl mercaptan, a garlic-derived organosulfur compound, inhibits histone deacetylase and enhances Sp3 binding on the P21WAF1 promoter." Carcinogenesis 29(9): 1816-1824.

[48] Druesne, N., et al. (2004). "Repetitive Treatments of Colon HT-29 Cells with Diallyl Disulfide Induce a Prolonged Hyperacetylation of Histone H3 K14." Annals of the New York Academy of Sciences 1030(1): 612-621

[49] Untergasser A, C. I., Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012). Primer3 - new capabilities and interfaces. Nucleic Acids Research. 40: 15.

[50] Willy, P. J., et al. (1995). "LXR, a nuclear receptor that defines a distinct retinoid response pathway." Genes Dev 9(9): 1033-1045.

[51] Wang, N., et al. (2000). "Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1." J Biol Chem 275(42): 33053-33058.

[52] Ye, Y.-C., et al. (2015). "Use of Atorvastatin in Lipid Disorders and Cardiovascular Disease in Chinese Patients." Chinese Medical Journal 128(2): 259-266.

[53] Kolawole, E. M., et al. (2016). "Fluvastatin Suppresses Mast Cell and Basophil IgE Responses: Genotype-Dependent Effects." Journal of immunology (Baltimore, Md. : 1950) 196(4): 1461-1470.

[54] Gebhardt, R., et al. (1994). "Inhibition of cholesterol biosynthesis by allicin and ajoene in rat hepatocytes and HepG2 cells." Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism 1213(1): 57-62.

[55] Sharpe, L. J. and A. J. Brown (2013). "Controlling cholesterol synthesis beyond 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR)." J Biol Chem 288(26): 18707-18715.

[56] Liao, Q. J., et al. (2009). "Effect of diallyl disulfide on cell cycle arrest of human colon cancer SW480 cells." Ai Zheng 28(2): 138-141.

[57] Amagase, H., et al. (2001). "Intake of garlic and its bioactive components." J Nutr 131(3s): 955s-962s.

[58] Zhao, J., et al. (2006). "Diallyl disulfide suppresses growth of HL-60 cell through increasing histone acetylation and p21WAF1 expression in vivo and in vitro." Acta Pharmacol Sin 27(11): 1459-1466.

[59] Spencer, T. E., et al. (1997). "Steroid receptor coactivator-1 is a histone acetyltransferase." Nature 389(6647): 194-198.

[60] Schiltz, R. L., et al. (1999). "Overlapping but distinct patterns of histone acetylation by the human coactivators p300 and PCAF within nucleosomal substrates." J Biol Chem 274(3): 1189-1192.

[61] Ikura, T., et al. (2000). "Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis." Cell 102(4): 463-473.

[62] Bao, Y., et al. (2009). "Identification of trichostatin A as a novel transcriptional up-regulator of scavenger receptor BI both in HepG2 and RAW 264.7 cells." Atherosclerosis 204(1): 127-135.

[63] Ashburner, B. P., et al. (2001). "The p65 (RelA) Subunit of NF-κB Interacts with the Histone Deacetylase (HDAC) Corepressors HDAC1 and HDAC2 To Negatively Regulate Gene Expression." Molecular and Cellular Biology 21(20): 7065-7077.

[64] Kim, H.-J. and S.-C. Bae (2011). "Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs." American Journal of Translational Research 3(2): 166-179.

[65] Jin, Y. H., et al. (2004). "Transforming growth factor-beta stimulates p300-dependent RUNX3 acetylation, which inhibits ubiquitination-mediated degradation." J Biol Chem 279(28): 29409-29417.

[66] Cao, J. Q., et al. (2014). "Histone Deacetylase 9 Represses Cholesterol Efflux and Alternatively Activated Macrophages in Atherosclerosis Development." Arteriosclerosis, Thrombosis, and Vascular Biology 34(9): 1871-1879.

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